147. Solid-Phase Synthesis of C-Terminal Peptide Amino Alcohols

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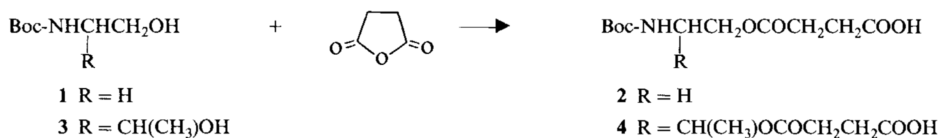
The solid-phase syntheses of enkephalin and somatostatin analogues with C-terminal OH functions instead of the normal carboxylates are described. The OH function of the N-terminal amino alcohol was acylated with succinic acid and esterified to the solid support. Normal Boc-TFA solid-phase strategy can be applied to build up and cleave these peptides. The succinic 'handle' can be removed by mild basic hydrolysis after cleavage.

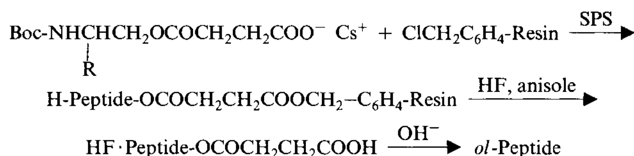
Introduction. – In the past, several highly interesting peptide analogues with C-terminal CH_2OH functions instead of the usual carboxylate have been reported. Among them, enkephalins [1] [2] as selective μ-receptor ligands [3] [4] and highly potent somatostatin analogues [5] are the typical representatives for this peptide class. The high potency of these peptide analogues is explained in part by their metabolic stability: it is proposed that the C-terminal CH_2OH function does not permit carboxypeptidase recognition. Therefore, degradation by this class of exopeptidases is strongly reduced. These *ol*-peptides, as they are frequently called, have all been synthesized by the solution method, where the absence of a C-terminal carboxylate or carboxamide has a relatively low importance. Growing interest in peptides displaying this C-terminal modification and the resulting need for analogue series would make it more convenient to proceed with solid-phase peptide synthesis (SPS) to obtain C-terminal CH_2OH peptide analogues. The absence of a standard C-terminal function does, however, not permit any of the currently used strategies, because all reactions for the immobilization of the C-terminal amino acid require a free carboxylate function. In the following, a chemical pathway is proposed and presented which will allow classical SPS for C-terminal CH_2OH peptide analogues.

In the classical SPS strategy, according to *Merrifield* [6], a N-terminally protected amino acid is esterified to chloromethylated polystyrene. In more recent approaches, a variety of linker functions with more or less acidolytic sensitivity have been described, but in every case, the C-terminal amino acid is always an acylating moiety. To be able to use the building blocks of the widely used Boc-TFA-HF strategy (TFA = trifluoroacetic acid) for SPS technology, the *N*-Boc-protected amino alcohol analogue of a given amino acid was first esterified with succinic anhydride, and the resulting hemi-succinate was then esterified to the resin by the usual Cs-salt method [7]. Chain elongation was then carried out as usual for the Boc-TFA scheme, and the completed peptide was cleaved from the resin with anhydrous HF. The resulting peptide-hemi-succinate was easily cleaved by mild saponification into the final CH_2OH analogue of the peptide (see *Schemes 1* and *2*):

Scheme 1



Scheme 2

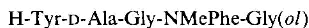


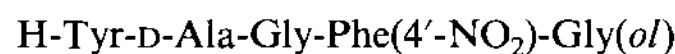
Results and Discussion. – The esterification of a succinic-acid linker to the *N*-protected amino alcohol (*Schemes 1* and *2*) brings this synthon back to the standard situation for SPS. The introduced linker was stable under the conditions used for the classical Boc-TFA scheme with 40% TFA in CH_2Cl_2, and the resin and side-chain cleavage conditions in liquid HF. The resulting peptide-hemi-succinates were cleaved by mild basic hydrolysis. The following peptides (see *Scheme 3*) were synthesized and compared to the commercially available products **5**, **6**, and **8**, analyzed by FAB-MS, HPLC, and TLC for their structural identity and purity.

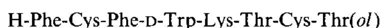
Peptide **5** is the μ-selective enkephalin analogue DAGO [4], peptide **7** is a potential photolabel with a structure similar to an already published photolabel with DAGO structure [8].

All peptides have been obtained in good yields without synthetic complications. The production of the *N*-Boc-amino-alcohol hemi-succinates succeeded in excellent yields. The attachment of the hemi-succinates by the Cs-salt procedure to the chosen support, chloromethylated polystyrene, was also uneventful. For the Boc-Thr(*ol*)-synthon, free

Scheme 3. *Amino-Acid Sequences of Amino Alcohol Analogues of Enkephalin and Somatostatin*







Gly(*ol*) = 2-aminoethanol; Thr(*ol*) = (2*R*,3*R*)-2-Aminobutane-1,3-diol; NMePhe = *N*-Methyl-L-phenylalanine.

hemi-succinates could not be detected after esterification to the resin, and it is concluded that this synthon was bound to the resin through both carboxylic functions.

After HF cleavage, traces of already desuccinylated peptide were detected, but most had to be hydrolyzed with NaOH/MeOH. The somatostatin analogue **8** was cyclyzed by hexacyanoferrate oxidation [9] before ester hydrolysis and purification. After saponification, purification, and lyophilization, all products had identical physico-chemical and biological properties as the commercial products, indicating the usefulness of this SPS approach for the synthesis of peptides containing C-terminal amino alcohols.

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Experimental Part

General. N-[(*tert*-Butoxy)carbonyl]amino acids (*N*-Boc-amino acids), and reference peptides **5** and **6** were obtained from *I.A.F. Biochemicals Inc.*, Montreal, peptide reagents from *Aldrich* or *Sigma*, chloromethylated polystyrene (co-polystyrene + 1% divinyl benzene; 0.75 mequiv./Cl per g) from *IAF-Biochemicals*, Montreal. (2*R*,3*R*)-2-aminobutane-1,3-diol and reference peptide **8** was a generous gift from *Sandoz AG*, Basel, Switzerland. *tert*-Butyl-1,2,2,2-tetrachloroethyl carbonate (Boc-TCE) was purchased from *Propeptide*, Vert-le-Petit, France. Anal. HPLC was carried out on a *Waters M45* instrument with *VYDAC 218 TP 104* reversed-phase columns and a 214-nm UV-detector. All solvents used for SPS were re-distilled before use. Gel filtration was carried out on *Sephadex G-15* columns, eluted with 0.25N $AcONH_4$ at pH 4.5. According to the applied peptide amount, different column dimension were used: above 1 g: 850 mm (length), 50 mm (diameter), between 1 g and 100 mg: 450 × 25 mm, below 100 mg: 350 × 13 mm. *Sephadex-LH-20* gel filtration was carried out in a 300 × 25 mm column, eluted with DMF. Prep. HPLC was carried out in *Michel-Miller* glass columns (10 × 150 mm or 20 × 300 mm) on reversed-phase material *Nucleosil 30 μ* (*Macherey Nagel*, Germany) and eluted with gradients of MeCN in 0.05% CF_3COOH (TFA), delivered at 8–10 atm with a FMI-lab pump, model *RP-G-1CSC, Fluid Metering Inc.*, Oyster Bay, N.Y.) through a 254-nm UV recorder (except for peptide **3**). Aliquots of the fractions after gel filtration or reversed-phase chromatography were spotted on TLC, the pure peptide fractions were combined and lyophilized. TLC was performed on *Merck DC-Alufolien Kieselgel 60 F_{254}* in the following solvent systems: *A*: $CHCl_3$/MeOH/AcOH 95:5:3; *B*: BuOH/AcOH/H_2O 5:2:3. Post-migration visualization was carried out first with UV fluorescence and then either with a modified *Reindel-Hoppe* procedure [10], ninhydrin, or bromocresol green. Peptides were considered pure, if the HPLC peptide-peak integral was at least 95% of the total combined peak integrals, and if they were homogenous in TLC. ^1H-NMR spectra were recorded on a *Varian 60* instrument in $CDCl_3$ soln. with TMS as internal standard ($\delta = 0$ ppm). Chemical shift δ is indicated in ppm, the coupling constant J in Hz. Peptide structure was confirmed by fast-atom-bombardment mass spectroscopy (FAB-MS, *Hewlett-Packard*, type *5988A*). FAB-MS samples were presented in thyoglycolic matrix.

*2-{[(*tert*-Butoxy)carbonyl]amino}ethanol* (**1**). Solid Boc-TCE (28,3 g, 0.1 mol) was slowly added, under vigorous stirring, to 50 ml of 2-aminoethanol at r.t. Stirring was continued overnight, and then 300 g of ice were added, followed by acidification to pH 3 with 1N HCl. The resulting 2-[*N*-Boc-amino]ethanol was extracted twice with AcOEt (200 and 100 ml), the combined org. phases were dried (anh. $MgSO_4$), and evaporated *in vacuo*: 16.2 g (99%) of an oily product. TLC: $R_f(A)$ 0.4. ^1H-NMR: 5.8 (br. *t*, $J = 5$, NH); 4.4 (br. *s*, OH); (*t*, $J = 5$, CH_2O); 3.25 (*m*, $J = 5$, CH_2N); 1.5 (*s*, *t*-Bu).

*2-{[(*tert*-Butoxy)carbonyl]amino}ethyl Succinate* (**2**). Compound **1** (10.2 g, 63 mmol) was dissolved in 20 ml of dry pyridine, and 9.5 g (95 mmol) of granular succinic anhydride was added under stirring at r.t. Stirring was continued overnight under exclusion of humidity. The volume was reduced *in vacuo*, 300 g of ice were added, and the mixture was acidified to pH 3 with 1N HCl. The product was extracted twice with AcOEt (300 and 100 ml), the org. fractions were combined, dried ($MgSO_4$), and evaporated *in vacuo*: 16.5 g (93%) of an oily product, which was purified by filtration over silica gel, eluted with $CHCl_3$/MeOH 4:1. The product-containing fractions were combined and evaporated, yielding an oil. TLC: $R_f(A)$ 0.63. ^1H-NMR: 4.15 (*t*, $J = 5$, CH_2O); 3.35 (*m*, $J = 5$, CH_2N); 2.68 (*s*, 2 CH_2); 1.5 (*s*, *t*-Bu).

*2-{[(*tert*-Butoxy)carbonyl]amino}butane-1,3-diol* (**3**). Solid Boc-TCE (45.8 g, 0.16 mol) was slowly added to a soln. of 9.58 g (91.2 mmol) of (2*R*,3*R*)-2-aminobutane-1,3-diol in 100 ml of *t*-BuOH, followed by 20 ml of dioxan to clear the soln. The reaction mixture was stirred overnight at r.t. and evaporated *in vacuo*. To the residue, 200 g of ice were added, and the mixture was extracted twice with 150 ml of AcOEt. The combined org. phases were dried ($MgSO_4$) and evaporated *in vacuo:* 10.1 g (53%) of **3** as an oil. TLC: $R_f(A)$ 0.28. ^{1}H-NMR: 5.05 (br. *t*, $J = 5$, NH); 4.4 (br. *s*, 20 H); 4.2–3.4 (br. *m*, 4 H); 1.5 (*s*, *t*-Bu); 1.25 (*m*, CH_3).

*2-{[(*tert*-Butoxy)carbonyl]amino}-1-methylpropan-1,3-diyldioxydicarbonyldipropionic Acid* (**4**). To 10 g (48 mmol) of **3**, dissolved in 80 ml of dry pyridine, were added 25 g (237 mmol) of succinic anhydride were added. Workup of the mixture as described above yielded 18.4 g (95%) of **4**. TLC: $R_f(A)$ 0.33; $R_f(B)$ 0.61. ^{1}H-NMR: 8.25 (*s*, 2 COOH); 5.05 (br. *m*, NH); 4.2–3.4 (br. *m*, 4 H); 2.68 (*s*, 2 CH_2CH_2).

Peptide Synthesis. The peptide syntheses were carried out with a *Peptomat* automatic peptide synthesizer, applying procedures described in [11]. The *N*-protected amino alcohols **1** and **3** were attached as the hemi-succinates **2** and **4**, respectively, to the resin by the Cs-salt procedure [7]. N^{α}-Boc protection was used for all amino acids. This temporary protecting group was removed, prior to the next coupling, by reaction with 40% CF_3COOH (TFA) in CH_2Cl_2 for 20 min at r.t. A 5% soln. of $EtN(i\text{-}Pr)_2$ in CH_2Cl_2 was used for the neutralization of the free amino function of the growing peptide. The ensuing coupling was performed with the symetrical anhydride of the next *N*-Boc-amino acid in a 4-to-6-fold excess [12]. The completion of every coupling was checked with the ninhydrin [13] test and the coupling repeated if necessary. After completion of the peptide sequence the last Boc-protecting group was cleaved, the resulting peptide resin salt washed, dried, and subjected to HF cleavage [14]. Peptide resin (1–5 g) was placed in a *Kel-F* reactor of an all *Teflon*-fluorocarbon cleavage instrument (*Peptomat* cleaver). As scavenger was added anisole (1.5 ml/g of resin), then, 10 ml of anh. HF were distilled into the reactor. The cleavage was carried out under slightly negative pressure and stirring with a magnetic stirrer at 0° for 1 h. The bulk of HF was flushed out by a stream of N_2, residual HF was removed by 2 × 15 min of evacuation through KOH pellets, first with a water aspiration pump, then under high vacuum. The peptides were extracted with 2N AcOH, lyophilized, and purified.

*H-Tyr-*D*-Ala-Gly-N-Me-Phe-NHCH$_2$CH$_2$OH* (**5**). The crude peptide (3.1 g), after HF cleavage of the peptide resin, was first purified by *Sephadex G-15* gel filtration, producing, after lyophilization of the main fraction, 1.73 g. On TLC (*B*) two products were detected with R_f 0.50 (main product) and R_f 0.63. This mixture (500 mg) was subjected to saponification in 4.5 ml of 1N NaOH in 7.5 ml of MeOH under stirring at r.t. for 3 h. After dilution with 30 ml of H_2O, the mixture was acidified with 2N AcOH, loaded directly onto a prep. reversed phase column, and eluted with a gradient of 5–45% MeCN: 231.7 mg of pure **5**. TLC: $R_f(B)$ 0.63, homogenous in HPLC. FAB-MS: 513 ([*M* + 1]). A sample of the crude peptide obtained by gel filtration was resolved by HPLC, and the product with R_f 0.63 was found to be identical to **5**. The product with R_f 0.50 had a FAB-MS with *M* + 1 peak at 613, corresponding to the hemi-succinate.

*H-Tyr-*D*-Ala-Gly-Phe-NHCH$_2$CH$_2$OH* (**6**). The crude peptide (1.06 g), after HF cleavage, was purified by *G-15* gel filtration. The product-containing fractions were combined and lyophilized, yielding 111.3 mg of product. This product (10 mg) was saponified and subjected to reversed-phase chromatography as described above, yielding 6.3 mg of **6**. TLC: $R_f(B)$ 0.52, homogenous in HPLC. FAB-MS: 499 ([*M* + 1]).

*H-Tyr-*D*-Ala-Gly-Phe(4′-NO$_2$)-NHCH$_2$OH* (**7**). After HF cleavage and lyophilization, 660 mg of this crude product were purified by *G-15* gel filtration; combining and lyophilization of the peptide-containing fractions yielded 109.9 mg of product. After hydrolysis and reversed-phase chromatography (as for **5**), 59.5 mg of pure **7** were obtained. TLC: $R_f(B)$ 0.55, homogenous in HPLC. FAB-MS: 545 ([*M* + 1]).

*H-Phe-Cys-Phe-*D*-Trp-Lys-Thr-Cys-NH-CH[CH(CH$_3$)OH]CH$_2$OH* (**8**). The completed peptide resin (3.35 g) was subjected as usual to HF cleavage but with 100 mg of D,L-*N*-Ac-Trp as additional scavenger. After extraction, the pH was adjusted to 8 with cautious addition of 4N aq. NH_3. The peptide was cyclyzed by addition of 600 mg of $K_3[Fe(CN)_6]$, followed by vigorous stirring at r.t. for 1 h. The mixture was lyophilized and then purified on *LH 20* in DMF. The peptide-containing fractions were united, partially evaporated *in vacuo*, diluted with H_2O, and lyophilized. The crude peptide was redissolved in 20 ml of H_2O and 2 ml of 0.5M Na-EDTA soln. at pH 9.5, charged onto a reversed-phase column, and washed with H_2O and finally with 0.25M $AcONH_4$ pH 9. The peptide was eluted with a gradient of 15–45% MeCN, the product-containing fractions were combined, partially evaporated *in vacuo*, diluted with H_2O, and lyophilized. A yield of 106.2 mg of product was obtained. Hydrolysis of 8 mg of this product yielded 4.4 mg of **8**, after purification on reversed-phase chromatography. TLC: $R_f(B)$ 0.51. FAB-MS: 1019 ([*M* + 1]). TLC and HPLC indicated homogeneity and identity to reference **8**.

REFERENCES

[1] D. Roemer, H. H. Buescher, R. C. Hill, J. Pless, W. Bauer, F. Cardinaux, A. Closse, D. Hauser, R. Hugenin, *Nature (London)* **1977,** *268,* 547.

[2] D. Roemer, J. Pless, *Life Sci.* **1979,** *24,* 621.

[3] H. W. Kosterlitz, *Proc. R. Soc. London, [Ser.] A* **1985,** *225,* 27.

[4] B. K. Handa, A. C. Lane, J. A. H. Lord, B. A. Morgan, M. J. Rance, C. F. C. Smith, *Eur. J. Pharmacol.* **1981,** *70,* 531.

[5] J. Pless, W. Bauer, U. Briner, W. Doepfner, P. Marbach, R. Maurer, T. J. Petcher, J.-C. Reubi, J. Vonderscher, *Scand. J. Gastroenterol.* **1986,** *21* (Suppl. 119), 54.

[6] R. B. Merrifield, *Adv. Enzymol.* **1969,** *32,* 221.

[7] B. Gisin, *Helv. Chim. Acta* **1973,** *56,* 1476.

[8] C. Garbay-Jaureguiberry, A. Robichon, B. P. Roques, *Int. J. Pept. Protein Res.* **1986,** *27,* 34.

[9] D. B. Hope, V. V. Murti, V. Duvigneaud, *J. Biol. Chem.* **1962,** *237,* 1563.

[10] E. Von Arx, M. Faupel, M. Brugger, *J. Chromatogr.* **1976,** *120,* 224.

[11] Z. Mackiewicz, S. Belisle, D. Bellarbara, N. Gallo-Payet, J.-G. Lehoux, E. Escher, *Helv. Chim. Acta* **1987,** *70,* 423.

[12] E. Wünsch, G. Wendelberger, *Chem. Ber.* **1967,** *100,* 160.

[13] E. Kaiser, R. L. Colecott, C. D. Bossinger, P. I. Cook, *Anal. Biochem.* **1970,** *34,* 595.

[14] S. Sakakibara, in 'Chemistry and Biochemistry of Amino Acids, Peptides and Proteins', Ed. B. Weinstein, M. Dekker, New York, 1971, Vol. 1, pp. 51–85.